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# MODIFICATION OF PROTEINS WITH COVALENT LIPIDS

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## I. INTRODUCTION

### A. *Historical*

In 1951, Folch-Pi and Lees described a protein from rat brain myelin that was soluble in organic solvents and insoluble in water.[34] This polypeptide, termed proteolipoprotein (PLP), was shown subsequently to contain covalently-bound fatty acid and represents the first fatty acid acylated protein to be identified.[2] The discovery of long chain fatty acids covalently associated with myelin PLP may have seemed initially to be a biochemical curiosity. However, subsequent searches for similar modifications on other membrane proteins revealed a broad array of polypeptides that contain covalent fatty acids, in addition to other lipid moieties.

Among the most common lipid species found to be associated with proteins are long chain saturated fatty acids. The 16-carbon saturated fatty acid, palmitate, has been identified on proteins anchored to the cytoplasmic surface of the plasma membrane and on several transmembrane glycoproteins. Myristate, the rare 14-carbon saturated fatty acid, also is attached to a subset of proteins that associate with the interior surface of the plasma membrane. Modification of proteins with these fatty acids is catalyzed by distinct sets of enzymes that exhibit unique substrate specificities and different subcellular distributions. A second form of protein lipidation involves the attachment of a complex glycosylated phosphatidylinositol (PI) moiety to the carboxy-terminus of polypeptide chains, where it functions as a hydrophobic anchor within the plasma membrane. While each of these types of lipid modifications are unique with respect to their structures and mechanisms involved in their attachment to proteins, they share the property of mediating the interactions of proteins with membranes. In several cases, the lipid moieties described above also have been demonstrated to influence diverse cellular processes, by virtue of their ability to modulate the function, and by appropriate intracellular sorting, of important

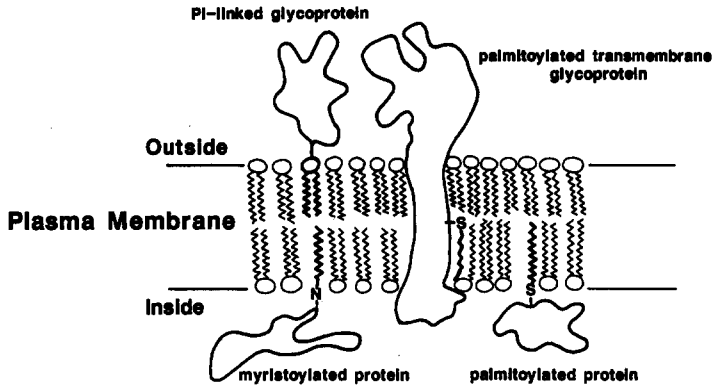


FIG. 1. Mechanisms whereby covalent lipids mediate the interactions of proteins with membranes. Palmitate is attached through an ester linkage to transmembrane glycoproteins, such as VSV-G glycoprotein, and to proteins that are anchored to the cytoplasmic surface of the plasma membrane, such as $p21^{ras}$. Myristate is attached through an amide linkage to otherwise soluble proteins, such as $p60^{src}$, and often mediates their interaction with membranes. A complex glycosyl-PI anchor is attached to the C-termini of several cell surface glycoproteins, such as acetylcholine esterase, and maintains their association with the bilayer.

regulatory proteins. A diagramatic representation of the above types of lipid modifications and the ways in which they influence the interactions of proteins with membranes is shown in Fig. 1.

The identification of proteins containing covalent lipids has necessitated the reevaluation of the generally accepted mechanisms for association of proteins with membranes. The well-characterized routes followed by integral membrane glycoproteins from their site of synthesis in the rough endoplasmic reticulum to their ultimate destinations in the plasma membrane, lysosomes, or other membrane systems now appear to represent only a subset of the pathways followed by membrane proteins within cells. Studies of proteins modified by covalent lipids have revealed new and novel mechanisms whereby proteins may interact tightly with membranes and undergo appropriate sorting within the cell.

### B. *Criteria for Identification of Covalent Lipid on Proteins*

In order for a protein to be considered to be modified by covalent lipid, several strict criteria should be met. The protein-associated lipid should be resistant to removal by boiling in SDS and by exhaustive extraction of the protein with organic solvents. The covalent lipid species also should be identified following its release from the polypeptide by chemical cleavage of the protein–lipid linkage.

The majority of fatty acylated proteins have been identified by the labeling of tissue culture cells with radioactive fatty acids, followed by separation on SDS-polyacrylamide gels and visualization by fluorography. Because less than 0.01% of lipids taken up by cells in tissue culture become incorporated into proteins, with the remainder entering the cellular lipid pools, it is essential to ensure that protein-labeling does not arise from residual noncovalently associated lipids. The latter issue is of particular importance, in light of the high affinity of many membrane proteins for lipids. Proteins modified by covalent lipids also represent a minor fraction of the total set of proteins within a cell, leading to further difficulties in their detection. An additional complication inherent in studies in which proteins are labeled *in vivo* with $^3$H-lipids is determination of the stoichiometry of lipidation. Because the specific activity of cellular lipid pools is difficult, if not impossible to determine, studies of this type often cannot distinguish whether a protein contains multiple sites for lipidation or alternatively, whether only a minor fraction of a particular polypeptide is modified at a single site.

As will be discussed in detail below, several different lipid moieties have been shown to be covalently associated with proteins. One cannot assume *a priori*, therefore, that a

radiolabeled precursor used to label tissue culture cells actually represents the species that ultimately becomes incorporated into the polypeptide. Long chain fatty acids, for example, may be elongated, desaturated, or incorporated into more complex lipid species prior to attachment to a polypeptide *in vivo*. Lipid precursors, used in metabolic labeling studies, also may be metabolically converted into other intermediates such as amino acids, carbohydrates, or acetate. In the absence of chemical identification of the covalently-associated lipid species, this latter caveat can lead to erroneous conclusions regarding the presence of covalent lipid on a protein. It is important, therefore, that the covalent lipid moiety be identified chemically.

## II. PROTEINS MODIFIED BY COVALENT FATTY ACIDS

### A. *Palmitoylated Proteins*

The most extensively studied class of proteins containing covalent lipids are those modified with long chain fatty acids.[68,101] The first member of this class to be identified was the myelin PLP, which represents a major protein component of myelin.[14,34,120] Agrawal and coworkers purified this polypeptide to homogeneity by extraction into chloroform/methanol (2:1). Subsequent analysis revealed the presence of 2 moles of covalent fatty acid per mole of PLP.[2,120] Palmitic acid was found to be the preferred acyl chain for this modification; however, lesser amounts of oleic and other long chain desaturated fatty acids were also associated with the protein.[9,120] *In vivo* labeling experiments in which $^3$H-fatty acid coupled to bovine serum albumin was injected into the brains of rats confirmed these early observations and resulted in identification of a second component of myelin, termed DM20, that was fatty acylated.[2]

Because of difficulties in studying the details of protein biogenesis in the intact animal, several years elapsed before the mechanisms involved in, and functions of, protein fatty acylation became readily accessible to experimental analysis. However, the discovery that fatty acylation is common to a wide range of viral proteins rendered these questions susceptible to investigation.

#### 1. *Structure of the Linkage Through Which Palmitate is Attached to Proteins*

The first lipid-containing proteins to be examined in detail were those containing palmitic acid. Proteins within this class are listed in Table 1. In the majority of palmitoylated proteins identified to date, the fatty acid is attached to the polypeptide through a thiol ester linkage to a cysteine residue. This type of linkage is extremely labile and can be hydrolyzed by mild alkali or by 1 M hydroxylamine at neutral pH. An exception to this type of palmitate linkage has been reported for brain PLP which contains palmitate, linked through an *O*-ester to a serine at position 198.[119]

Using $^3$H-acetate as a precursor, Towler and Glaser reported that palmitate, synthesized *de novo* by the $BC_3H1$ and A431 cell lines, was incorporated preferentially in proteins through thiol ester linkages.[124] However, ca. 30% of protein-associated palmitate was resistant to removal by hydroxylamine, indicating the existence of a minor pathway for incorporation of palmitate into proteins through amide linkages. Using exogenous palmitate to label these cells, less than 10% of the fatty acid became incorporated into proteins through an amide linkage. These results suggest that endogenously synthesized palmitate, released from the fatty acid synthetase, is the donor for attaching palmitate to proteins in amide linkage. The identity of the amino acid acceptor for this form of protein acylation was not determined nor were the identities of the proteins labeled through palmitate amide linkages established.

$^3$H-palmitate also has been reported to label the nicotinic acetylcholine (ACh) receptor[76] through a linkage insensitive to hydrolysis by standard methods that release ester-linked lipids, suggesting that this cell surface glycoprotein may be among the class of acyl proteins containing amide-linked palmitate. The actual identity of the fatty acid associated with

TABLE 1. Proteins Modified by Covalent Palmitic Acids

| Protein | Reference |
|---|---|
| $p21^{ras}$ | 21, 23, 106 |
| Sodium channel | 99 |
| $Ca^{2+}$ ATPase | 65 |
| Mammalian transferrin receptor | 79 |
| Human gastric mucus glycoprotein | 112–115 |
| Rhodopsin | 75 |
| Ankyrin | 118 |
| Apolipoprotein A-I | 45 |
| Major histocompatability complex antigens | 52 |
| Murine Ia $\alpha$, $\beta$ and invariant chains | 110 |
| Vinculin | 16 |
| Nicotinic acetylcholine receptor | 76 |
| Brain myelin proteolipoprotein | 2, 34 |
| Milk fat globule membrane butyrophilion and xanthine oxidase | 53 |
| Simian virus 40 large T antigen | 44, 56 |
| Vesicular stomatitis glycoprotein | 103 |
| Semliki Forest virus glycoproteins | 100 |
| Sindbis virus glycoproteins | 101 |
| Influenza virus hemagglutinin $Ha_2$ | 101 |
| Fowl-plague hemagglutinin $H_2$ | 101 |
| Newcastle disease virus fusion glycoprotein F1 | 101 |
| Corona virus glycoprotein E2 | 72, 101 |
| LaCross virus glycoprotein G1 and G2 | 101 |

this receptor was not determined, however, leaving open the possibility that the palmitate may have been metabolically converted to another lipid species.

Despite the identification of a relatively large number of palmitate-containing proteins, the actual amino acid residues that serve as acylation sites have been determined in only a few cases. The difficulties in identifying acylated amino acids within this class of acyl proteins are due, at least in part, to the hydrophobic nature of acylated protein domains, in addition to the low level of expression of most cellular acyl proteins. The extreme lability of palmitoyl ester linkages also contributes to difficulties in identification of acylated cysteine residues. In vescicular stomatitis virus (VSV)-G-protein,[95] the transferrin receptor,[49] and the HLA glycoprotein,[52] the sites of palmitoylation have been localized to cysteine residues within the transmembrane domains of the polypeptides. $p21^{ras}$ also contains palmitate, linked to a cysteine located four residues from the carboxy terminus.[23] Comparison of the amino acid sequences surrounding the acylation sites in these proteins does not reveal obvious homologies or structural similarities that might serve as a recognition sequence for a palmitoyl acyltransferase. Detailed analysis of the structural requirements for palmitoylation using synthetic peptides, as has now been performed for myristoylation (see below), has not yet been undertaken.

## 2. *Biogenesis of Palmitoylated Proteins*

(*a*) *Palmitoylation of cell surface glycoproteins.* Initial studies designed to identify and characterize palmitoyl acyltransferase(s) were carried out using chicken embryo fibroblasts infected with Sindbis and Vescicular Stomatitis viruses, each of which encodes a transmembrane fatty acylated glycoprotein within its genome. Envelope virus glycoproteins were particularly valuable as a model for exploring the biosynthesis of palmitoylated proteins because these membrane proteins are expressed at levels up to 1000-fold higher than normal cellular proteins and because viral infection results in the arrest of cellular protein synthesis, thereby leading to a simplified system for analysis. The availability of the complete amino acid sequence of several envelope virus glycoproteins, combined with the existence of mutants with defined structural alterations in different domains of these proteins (see below), also facilitated analysis of the structural requirements for acylation.

Using VSV- and Sindbis virus-infected fibroblasts, Schmidt and Schlesinger demon-

strated that inhibition of protein synthesis with cycloheximide prevented palmitate acylation of virus glycoproteins following a lag of 5 to 10 min.[103] Newly acylated glycoproteins were sensitive to digestion with endo-$\beta$-*N*-acetylglucosaminidase H (Endo H), indicating that they were modified by high mannose simple-type oligosaccharide side chains. By 20 to 30 min after synthesis, viral glycoproteins became Endo H-resistant, marking their conversion to complex glycoproteins in the medial stacks of the Golgi apparatus.The acquisition of Endo-H-resistance occurred 3 to 6 min after acylation, suggesting that the palmitoyl acyltransferase is located within the *cis*-Golgi or transitional elements of the endoplasmic reticulum.[103] This conclusion is supported by the observations of Dunphy *et al.*[28] and Quinn *et al.*,[89] who showed that addition of fatty acids to G-protein occurred in a smooth membrane fraction that contains oligosaccharide-trimming activity. Inhibition of *N*-linked glycosylation of VSV-G and Sindbis virus glycoproteins in the presence of tunicamycin prevents their transport to the cell surface and abolishes palmitoylation, presumably because newly synthesized viral proteins do not reach the subcellular compartment that contains the acylating activity.[102] Together, these results suggest that acylation of glycoproteins is restricted to an early compartment in the transport pathway of these proteins to the cell surface. The ability of protein synthesis inhibitors to block palmitoylation of the envelope virus glycoproteins also indicates that these proteins do not undergo reversible acylation–deacylation, because turnover of the lipid group would be expected to occur independently of protein synthesis.

The transferrin receptor was among the first cellular fatty acylated proteins to be examined in detail. Using an approach similar to that described above for the envelope virus glycoproteins, Omary and Trowbridge showed that the transferrin receptor acquired covalent palmitic acid with different kinetics from those of the viral glycoproteins.[79,80] Labeling of the transferrin receptor with $^3$H-palmitate appears to be a late post-translational event that continues unabated for up to 48 hr following inhibition of protein synthesis with cycloheximide. In the presence of tunicamycin, the transferrin receptor is transported to the cell surface, albeit inefficiently, and undergoes acylation. Comparison of the turnover rate of the polypeptide chain and the covalent lipid moiety revealed that the lipid turns over at a rate ca. 4-fold faster than the protein to which it is attached. Although there are inherent complications in comparing turnover rates of polypeptides and lipids in pulse-labeled cells, these results suggest that the transferrin receptor may undergo a cycle of reversible acylation–deacylation–reacylation. It is interesting that this glycoprotein is among the receptors that recycles continuously from the cell surface to the interior of the cell, raising the possibility that an acylation–deacylation cycle might be involved in receptor recycling. Internalization, for example, might bring the transferrin receptor into an intracellular compartment where it can undergo deacylation and reacylation. Alternatively, the entire deacylation–reacylation cycle might occur entirely within the plasma membrane. As will be discussed throughout this review, experimental evidence from diverse systems suggests the existence of multiple protein acyltransferases, therefore, there is no reason to assume *a priori* that the same enzyme is responsible for the initial acylation of the transferrin receptor in the Golgi apparatus and its subsequent acylation after reaching the cell surface.

In addition to these well-characterized glycoproteins that undergo palmitoylation, a broad array of at least 20 cellular glycoproteins, whose identities have not yet been determined, have been observed by labeling tissue culture cells with $^3$H-palmitate followed by analysis on SDS-polyacrylamide gels.[66,70,77,78,97] Palmitate incorporation into at least one secreted protein, apolipoprotein C, also has been reported.[45] This polypeptide is transported and processed through the secretory pathway and may, therefore, acquire its covalent lipid group by interaction with the same enzyme(s) in the *cis*-Golgi apparatus that acylate cell surface glycoproteins.

(*b*) *Palmitoylation of nonglycosylated membrane proteins.* Although cell surface glycoproteins initially were the most thoroughly characterized palmitoylated proteins, they do not represent the only class of proteins modified with this fatty acid. A second set of

palmitoylated proteins lacks carbohydrate and follows intracellular pathways distinct from the palmitate-containing glycoproteins.

PLP,[2,14,34,120] $p21^{ras}$,[23] and ankyrin,[117,118] for example, acquire covalent fatty acid during their maturation and are nonglycosylated. Both $p21^{ras}$ and ankyrin are translated on free polysomes and associate with the cytoplasmic surface of the plasma membrane soon after synthesis. The kinetics for palmitoylation of *ras* coincide with the timing for membrane binding; however, it remains to be established definitively whether this modification occurs immediately prior to, or following, its interaction with membranes.[67,106] As will be discussed in greater detail below, indirect experimental evidence favors the addition of palmitate to these proteins in the cytosol as a prerequisite for membrane association. In sharp contrast to the tightly constrained kinetics for palmitoylation of the envelope virus glycoproteins, palmitate incorporation into *ras* and ankyrin occurs following inhibition of protein synthesis with cycloheximide.[67,117] Ankyrin also can be labeled with $^3$H-palmitate in mature erythrocytes, which do not synthesize protein,[118] indicating that its synthesis and acylation are not coupled. As was found for the transferrin receptor, the lipid moiety attached to *ras* and ankyrin appears to turn over rapidly, which would explain the observation that palmitate can be incorporated into these polypeptides for several hours following inhibition of protein synthesis.

In an effort to determine the relative prevalence of the different pathways for palmitoylation, the characteristics of palmitate-containing proteins have been examined in tissue culture cells. Using this approach, Olson and Spizz estimated that less than 1% of palmitate-containing proteins in $BC_3H1$ muscle cells were transmembrane glycoproteins, which would be expected to be processed through the secretory pathway.[77] The remainder of cellular palmitoylated proteins did not contain detectable carbohydrate and were acylated through a mechanism independent of protein synthesis. Subcellular factionation studies demonstrated that this class of palmitoylated proteins was localized exclusively to the plasma membrane where they were resistant to release by techniques that strip peripheral proteins from membranes.[134] Proteolytic digestion of intact cells also was shown to have no effect on the mobility of these proteins on SDS-polyacrylamide gels, whereas the same treatment of isolated membranes led to their digestion.[134] Together, these studies indicate the existence of a major class of palmitoylated proteins that are bound tightly to the cytoplasmic surface of the plasma membrane. These proteins follow intracellular pathways distinct from cell surface glycoproteins and may be acylated by a different acyltransferase. To date, the existence of palmitoylated proteins in the cytosol has not been reported.

(*c*) *Nonenzymatic palmitoylation.* The extremely labile nature of the thiol ester linkages through which palmitate is attached to proteins can lead to difficulties in analysis of palmitoylated proteins. Because most studies of protein fatty acylation involve analysis of metabolically labeled polypeptides on SDS-polyacrylamide gels under denaturing conditions, significant loss of label can occur during prolonged heating of samples in the presence of reducing agents. Perhaps more problematic is the potential for nonenzymatic exchange of fatty acids between Coenzyme A and free sulphydryl groups on proteins. This phenomenon becomes very important in analysis of cell-free palmitoylation of proteins (see below) and can lead to erroneous conclusions regarding the presence of covalent fatty acids on proteins *in vivo*.

The potential for nonenzymatic acylation is illustrated by studies on rhodopsin. O'Brien and Zatz demonstrated that bovine retinal rod outer segments support the incorporation of palmitate into this protein via a mechanism insensitive to inhibition of protein synthesis.[75] Subsequent analysis of the characteristics of this modification revealed that rhodopsin was palmitoylated through a nonenzymatic thiol ester transfer reaction that appeared to utilize palmitoyl-CoA as the acyl donor.[74] This reaction apparently occurs *in vivo* as well as *in vitro*, which raises questions as to its significance. Such studies reinforce the importance of exercising caution in interpretation of results that indicate that proteins are acylated with fatty acids through thiol ester linkages.

(*d*) *Reconstitution of palmitoylation* in vitro. The apparent complexity of palmitoylation suggests the existence of multiple palmitoyl acyltransferases that reside in distinct subcellular locations and exhibit different substrate specificities. Attempts to purify such acyltransferases have, thus far, been unsuccessful. However, a number of laboratories have established *in vitro* systems for protein palmitoylation that will undoubtedly lead to the eventual purification and characterization of the enzyme(s) responsible for this modification.

Incubation of rat brain myelin with palmitoyl-CoA has been shown to result in labeling of the major PLP and the proteolipid, DM-20.[10,11,137] Palmitate was attached to the myelin proteins through an ester linkage *in vitro* and was demonstrated through peptide mapping to be attached to the same site on PLP as occurs *in vivo*. Pulse-chase experiments indicated that the reaction involved the net addition of fatty acid to the PLP and not rapid fatty acid exchange. The $K_m$ and $V_{max}$ for palmitoyl-, stearoyl- and oleoyl-CoA were similar, whereas myristoyl-CoA was a less efficient substrate for the reaction. Acylation of PLP *in vitro* also proceeded with linear kinetics for 30 min before leveling off, suggesting the existence of a limiting pool of nonacylated PLP in myelin. The latter observation is consistent with the continued palmitoylation of PLP in brain tissue slices following inhibition of protein synthesis. The ability of myelin to support the enzymatic palmitoylation of proteolipoproteins also indicates that the fatty acyltransferase responsible for this reaction is localized to myelin and might, therefore, be distinct from the acyltransferase in the Golgi apparatus that acylates glycoproteins *en route* to the cell surface or the enzyme that acylates nonglycosylated proteins outside of the secretory pathway.

Similar studies have been carried out using envelope virus glycoproteins as substrates for palmitoylation *in vitro*. Exogenous VSV-G-protein, obtained by extraction of mature virus particles, can be acylated *in vitro* by intracellular membrane fractions from a variety of cell types only after it has been deacylated with hydroxylamine.[6,64] Fatty acid transferred to G-protein *in vitro* is sensitive to release from the polypeptide by treatment with hydroxylamine, indicating that the palmitoyl acyltransferase retains its specificity for ester linkages. Cell free transfer of fatty acid to G-protein also has been observed using endogenous G-protein present in membrane fractions from virus-infected cells.[64] In this case, fatty acids become linked to a G species that has not undergone terminal glycosylation and has, therefore, not entered the trans-compartment of the Golgi apparatus. Palmitoylation of VSV-G-protein *in vitro* requires ATP; other nucleoside triphosphates cannot substitute. The ATP requirement can be eliminated, however, in the presence of palmitoyl-CoA as a lipid donor. Thus, the biochemical conditions for the transfer of $^3$H-palmitic acid to G-protein *in vitro* are very similar to those *in vivo*. A major exception, however, is that myristic acid serves as an efficient lipid donor *in vitro*, whereas this rare fatty acid is not incorporated into G-protein *in vivo*. These results suggest that the fatty acyl chain specificity *in vivo* may be dictated by the high abundance of palmitate relative to myristate, thereby making palmitate the more efficient lipid donor for the reaction. Using deacylated Semliki Forest Virus E1 glycoprotein as a substrate for acylation *in vitro*, the protein fatty acyltransferase also was shown to exhibit properties of an integral membrane protein, with no activity being detectable in the cytosol of tissue culture cells. Subcellular fractionation studies revealed that the enzyme was localized to the rough endoplasmic reticulum.[7]

A fatty acyltransferase from rat gastric mucosa that catalyzes the transfer of palmitic acid to gastric mucous glycoprotein also has been demonstrated *in vitro*. This enzymatic activity is apparently enriched in a Golgi membrane, although the potential presence of plasma membrane and rough endoplasmic reticulum within this fraction were not examined.[113] Optimum activity was observed using palmitoyl-CoA as the lipid donor; however, the potential ability of other long chain fatty acyl CoA's to serve as substrates for the reaction was not reported. Similarly, acylation of the transferrin receptor has been demonstrated *in vitro* using sheep reticulocytes.[1] Incorporation of fatty acid is dependent on ATP and the fatty is 5–10 times less effective as an acyl donor than the acyl CoA

derivative. $^{3}$H-myristic acid was used as the substrate for the *in vitro* reaction, rather than palmitate, which is the preferred substrate *in vivo*. Therefore conclusions regarding fatty acyl chain specificity of the reaction cannot easily be drawn. Nonetheless, the ability of myristate to acylate the transferrin receptor suggests that the acyl chain specificity observed *in vivo* may have been lost in the reconstituted system. $p21^{ras}$, which has been deacylated by hydroxylamine treatment, also has been reported to be acylated in the presence of a crude microsomal preparation through an enzyme-mediated reaction that utilizes palmitoyl-CoA as an acyl donor.[36]

Incorporation of $^{3}$H-palmitate and other fatty acids into cellular proteins, whose identities are unknown, has also been examined. Incubation of cell-free homogenates of heart, liver, or kidney with either $^{3}$H-palmitate or $^{3}$H-myristate plus ATP, or the corresponding $^{3}$H-fatty acyl CoA's results in acylation of several membrane proteins through an ester linkage.[91] Specific acylation is not detected in cell extracts that have been boiled prior to incubation with labeled fatty acids, suggesting that the acylation reaction is enzyme-mediated. Some polypeptides are acylated transiently under these conditions, raising the possibility that these proteins might serve as long chain acyl donors and thereby function as potential intermediates in the transfer of acyl chains to specific acceptors. It is presently unknown whether each of these palmitoyl acyltransferase activities represent the same, or different, enzymes.

As discussed above for palmitoylation *in vivo*, studies on *in vitro* acylation of proteins through ester linkages are subject to complications from nonenzymatic acyl exchange. At pH values above 8.5, for example, a dramatic increase in the amount of fatty acid incorporation into proteins *in vitro*[11] is observed. Moreover, proteins that are normally not acylated, such as albumin, carbonic anhydrase, and alcohol dehydrogenase can be labeled using $^{3}$H-fatty acyl CoA's at pH 11, but not at pH 7.5, due to the labile nature of the thiol ester linkages. In studies of acylation *in vitro*, it is essential, therefore, to demonstrate that esterification of fatty acids to proteins shows an optimum at 37°C and is inhibited by boiling or by protein-denaturation.

In addition to protein fatty acyltransferase activity, a protein fatty acylesterase activity that cleaves esterified fatty acids from proteins *in vitro* has been reported.[8] This "deacylase" is present in microsomal membranes from baby hamster kidney cells, pig liver, calf kidney and human mucous cells. The enzyme removes fatty acid from endogenous and exogenous viral acyl protein substrates and requires the presence of nonionic detergents for activity *in vitro*. Partial purification of the fatty acylesterase indicates that it is distinct from protein fatty acyltransferase operating in the reverse direction. The existence of an enzyme that removes palmitate from proteins is consistent with the observation that some palmitoylated glycoproteins, such as the transferin receptor, undergo rapid turnover of the fatty acid moiety.

### 3. *Functions for Palmitoylation*

As has been the case for most covalent modifications of proteins, determining the function for palmitoylation has proven to be a more arduous task than defining the structural or temporal nature of the modification. One reason for difficulties in determining the function of covalent palmitate on proteins has been the lack of specific inhibitors of this modification. The isolation or construction of proteins bearing mutations at, or surrounding, the site for palmitate attachment, however, has enabled some conclusions to be reached concerning the role for this modification.

The initial discovery of palmitoylation as an early post-translational modification common to the envelope virus glycoproteins led to speculation concerning the potential of this modification as a signal for targeting to the plasma membrane. The fact that only a minor fraction of cell surface glycoproteins contains covalent palmitic acid, however, indicates that this modification is not a prerequisite for targeting proteins within the secretory pathway to the plasma membrane. Site-specific mutation of the cysteine residue

that is normally palmitoylated in VSV-G glycoprotein also does not interfere with expression of the protein on the cell surface or its incorporation into virions.[95] The functions, if any, of palmitate on glycoproteins, therefore, may be very subtle and difficult to establish.

Petri *et al.* investigated the potential influence of covalent lipid on the properties of the lipid bilayer surrounding glycoproteins using fluorescently labeled VSV-G-protein, obtained by growing virus-infected hamster kidney cells in the presence of 16(9-anthroyloxy) palmitate.[88] Labeled G-proteins were reconstituted into dipalmitoyl-phosphatidylcholine vesicles and the mobility of the protein-bound fatty acid was determined by fluorescence measurements as a function of temperature. These studies indicated that the covalent lipid moiety interacted strongly with the surrounding bilayer, resulting in the removal from the phase transition of phospholipid in the region of G-protein. These results deomonstrate that the acylation site on G-protein is orientated such that it interacts directly with the membrane and that the covalent fatty acid is associated with the bilayer rather than sequestered within the secondary structure of the polypeptide.

The functions of covalent palmitic acid on the human gastric mucous glycoprotein also have been examined by Slomiany and coworkers.[114-115] Purified mucous glycoprotein is resistant to digestion by pronase, whereas following removal of covalently-associated palmitate, by treatment with hydroxylamine, the glycoprotein becomes pronase-sensitive. Mucous glycoprotein isolated from patients with cystic fibrosis is highly resistant to pronase and contains two- to three-fold more covalent fatty acid per mole than the normal protein. These results suggest that the covalent fatty acid might somehow protect the protein from proteolytic digestion, although the mechanism whereby this might occur is unclear. The apparent protease-resistance of the mucous glycoprotein in cystic fibrosis has been speculated to interfere with its normal turnover and to contribute to the abnormal accumulation of poorly soluble secretions associated with the disease.

The 12-carbon fatty acid amide, cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienoylamide) which inhibits *de novo* fatty acid synthesis by binding irreversibly to the $\beta$-ketoacyl acyl carrier protein synthetase was reported by Schlesinger and Malfer to inhibit incorporation of exogenous palmitate into VSV-G protein.[98] In the presence of cerulenin, nonacylated G-protein was transported normally to the cell surface but was not assembled into virions. In $BC_3H1$ muscle cells, cerulenin also partially inhibits assembly of acetylcholine (ACh) receptor subunits into a multi-subunit receptor complex, a process that normally occurs in the Golgi apparatus.[76] Although the above studies suggest that palmitoylation may influence protein–protein or protein–lipid interactions within membranes, they should be interpreted with caution because cerulenin may have nonspecific effects on cells. The mechanism whereby cerulenin inhibits acylation of proteins also is unclear. In the case of the $\alpha$ subunit of the ACh receptor, palmitoylation occurs through an amide linkage,[76] which Towler and Glaser showed was coupled to *de novo* fatty acid synthesis.[124] Cerulenin might, therefore, interfere with $\alpha$-subunit acylation indirectly, by inhibiting fatty acid synthesis.

Studies of the GTP-binding protein, $p21^{ras}$, suggest that palmitoylation is required for association of some otherwise soluble proteins with membranes. Mutations that replace or eliminate the carboxy-terminal domain of p21 that contains cysteine 186, the site of palmitoylation, prevent acylation and result in a loss in the ability of the protein to interact tightly with membranes.[135,136] These nonacylated mutants bind GTP normally, but fail to induce cellular transformation. Thus, it can be concluded that either palmitoylation or membrane association, or both, are key steps in the process whereby *ras* transforms cells. These results should be interpreted with some caution, because it cannot yet be concluded whether mutations at the carboxy-terminus prevent palmitoylation and thereby prevent membrane binding, or alternatively, prevent membrane binding which consequently prevents palmitoylation. Identification and subcellular localization of the enzyme(s) responsible for palmitoylation of $p21^{ras}$ should help distinguish between these possibilities.

In the cases where covalent palmitate appears to influence the behavior of proteins, it also remains to be established whether any fatty acid attached at the same site will have

the same biological effect or whether palmitate confers unique properties on proteins to which it is attached.

## B. *Myristoylated Proteins*

A second class of fatty acylated proteins that has received intense scrutiny in recent years contains covalent myristic acid. The first proteins to be demonstrated to contain myristic acid were calcineurin B[3] and the catalytic subunit of the cyclic AMP-dependent protein kinase from bovine cardiac muscle.[22] Initial attempts to identify the amino-terminal amino acids of these proteins by Edman degradation were unsuccessful due to blocked amino termini. Structural identification of the amino-terminal amino acid and its blocking group ultimately revealed that myristic acid was attached through an amide linkage to the $\alpha$-amino group of glycine at the *N*-terminus of both proteins. Since the initial identification of this form of fatty acylation, a wide range of proteins of viral and cellular origin have been shown to be modified by acylation with myristic acid. Proteins containing covalent myristic acid are shown in Table 2.

### 1. *Structure of the Linkage Through Which Myristate is Attached to Proteins*

In contrast to the labile nature of the linkage through which palmitate is attached to most proteins, myristate is highly specific for amide linkages, which are extremely stable and require acid hydrolysis to release the fatty acid. Chemical identification of the acylated amino acids in myristoylated proteins have revealed glycine as the acceptor for the fatty acid in all cases. Initial comparisons of the amino acids adjacent to the *N*-terminal glycine of myristoylated proteins failed to reveal any obvious consensus sequence for this modification such as the Asn-X-Thr/Ser acceptor sequence for *N*-linked glycosylation.[132] Nevertheless, it seemed that some type of structural specificity for this modification must exist because only a fraction of polypeptides containing *N*-terminal glycine were myristoylated. Moreover, mutagenesis of amino acids near the *N*-terminus, that left the essential glycine unchanged, abolished myristoylation, indicating that only certain residues were compatible with this modification.

Using a series of synthetic peptides as substrates for myristoylation *in vitro*, Towler *et al.* defined the amino-terminal sequence requirements for myristoylation.[125–127] The *N*-myristoyl acyltransferase (NMT) that transfers myristate to proteins is absolutely specific

TABLE 2. Proteins Modified by Covalent Myristic Acid

| Protein | Reference |
|---|---|
| Catalytic subunit of cAMP-dependent protein kinase | 22 |
| Calcineurin b | 3 |
| NADH cytochrome b5 reductase | 82 |
| $p60^{src}$ | 20, 104 |
| LSTRA T-cell lymphoma kinase $p56^{lck}$ | 69, 130 |
| $p120^{-abl}$ transforming protein | 105 |
| $p85^{-fes}$ transforming protein | 105 |
| $p29^{-ras}$ transforming protein | 105 |
| Picornavirus capsid protein VP4 | 85 |
| Polyoma virus and Simian virus 40 capsid protein VP2 | 121 |
| $\alpha$-Subunits of guanine nucleotide binding proteins | 19 |
| Pre-S1 protein of Hepatitus V virus | 87 |
| *Gag* proteins | |
| p15 of Maloney murine leukemia virus | 43 |
| p15 of Feline sarcoma virus | 41, 105 |
| p12 of Baboon endogenous virus | 105, 123 |
| p19 of Adult T-cell leukemia virus | 81, 107 |
| p17 of Human immunodeficiency virus | 131 |
| p14 of Bovine leukemia virus | 94 |
| p12 of Avian reticuloendotheliosis virus | 105 |
| p15 of Human T-cell leukemia virus type II | 81, 109 |
| p10 of Mouse mammary tumor virus | 105 |
| p10 of Mason-Pfizer monkey virus | 105 |

for glycine at the *N*-terminus. Peptides with Asn, Gln, Ser, Val, or Leu as the residue penultimate to Gly are effective substrates for myristoylation, whereas peptides with Asp, D-Asn, Phe or Tyr at this position are not myristoylated. Ala, Ser, or Glu residues at the third position are also effective substrates for the enzyme. Each of these structural requirements for myristoylation *in vitro* is compatible with the structures of the *N*-termini of proteins known to be myristoylated *in vivo*. A particularly interesting aspect of the specificity of NMT for substrates *in vitro* is the observation that peptides containing aromatic amino acids adjacent to glycine competitively inhibit NMT. Such peptides bind to the enzyme, but are not acylated. As suggested by Towler *et al.*,[126] peptides of this type might permit selective inhibition of NMT *in vivo*. It should be pointed out that additional NMT's with different substrate specificities also may exist, but would have gone undetected using the purification and assay strategy employed by Towler and coworkers.

## 2. *Biogenesis of Myristoylated Proteins*

During the past year, the biosynthetic steps involved in protein myristoylation have been defined in considerable detail. The more rapid progress in establishing the mechanisms involved in myristoylation, relative to palmitoylation, can be attributed, at least in part, to the highly stable nature of the linkage through which myristate is attached to proteins and to the observation that NMT does not behave in the same manner as integral membrane proteins, which are often difficult to purify due to their hydrophobicity.

NMT has been purified to homogeneity from yeast *Saccharomyces cerevisiae* and partially purified from $BC_3H1$ cells.[126,127] The native enzyme exhibits an apparent $M_r = 55$ kDa, exhibits no divalent cation requirements, and appears to contain a histidine residue essential for enzymatic activity. The enzyme exhibits a remarkable specificity for myristoyl-CoA as the acyl donor; decanoyl- and dodecanoyl-CoA are significantly less effective substrates, while palmitoyl-CoA is virtually inactive as an acyl donor. This acyl chain specificity is apparently attributable to the catalytic selectivity of NMT, because acyl-CoA's of varying chain length and desaturation are bound by NMT. Because myristic acid accounts for only 1–3% of the fatty acid content of eucaryotic cells,[54,55] with decanate and dodecanoate being represented in only trace amounts, the substrate specificity of the enzyme is sufficient to account for the exclusive presence of myristate on the *N*-termini of appropriate polypeptide chains *in vivo*.

The precise subcellular localization of NMT has not yet been determined, but, the enzyme is present in both crude membrane and soluble fractions prepared from yeast and from $BC_3H1$ cells,[125] suggesting that it may behave as a peripheral membrane protein or may be associated with polysomes (see below). The fact that many myristoylated proteins are translated on free polysomes indicates that the NMT is not restricted to the secretory pathway as appears to be the case for some palmitoyl acyltransferases. Interestingly, the amino terminus of NMT is blocked to Edman degradation; however, the identity of the blocking group has not yet been reported.[127] It will be of particular interest to determine whether NMT is itself myristoylated.

The temporal nature of myristoylation was established initially by determining the sensitivity of this modification to inhibition of protein synthesis. Several groups demonstrated independently that inhibition of protein synthesis abolished $^3$H-myristate labeling of proteins in tissue culture cells.[18,66,70,77] These studies indicated that myristoylation was an extremely early modification that occurred within seconds to minutes following polypeptide synthesis. The observation that myristoylation is completely abolished by inhibition of protein synthesis indicates that cells do not normally contain a pool of nonacylated proteins that can acquire myristic acid at different times after their synthesis. These results also imply that the myristate moiety is highly stable and does not turn over independently from the polypeptide, as appears to be the case for some palmitoylated proteins.

Studies using inhibitors of protein synthesis cannot distinguish whether addition of myristate occurs during, or within seconds following, translation of the acylprotein

polypeptide chain. Therefore, to define precisely when myristate is attached to newly synthesized acylproteins, Wilcox *et al.* examined whether nascent polypeptide chains are modified by myristic acid.[133] Nascent chains were isolated from $BC_3H1$ cells following pulse-labeling with $^3H$-myristate for 10 min. By this technique, myristic acid was shown to be attached to nascent polypeptide chains through an amide linkage and was identified by thin layer chromatography after its release from nascent chains by acid methanolysis. Inhibition of cellular protein synthesis with puromycin prevented incorporation of myristic acid into nascent chains, in agreement with the dependence of this modification on protein synthesis *in vivo*. These data represent a direct demonstration that myristoylation of proteins is a cotranslational modification.

In contrast to palmitoylated proteins, which appear to be located exclusively on membranes, myristoylated proteins exhibit a more widespread subcellular distribution. Several myristate-containing proteins are localized to the cytosol and do not appear to associate with membranes, while others are tightly bound to membranes.[18,66,70,77,78] Finally, other myristoylated proteins such as calcineurin b are distributed in membrane and cytosolic fractions. Myristate is rarely, if ever, associated with transmembrane glycoproteins. The majority of membrane-bound myristoylated proteins interact tightly with the bilayer and cannot be released by conditions that remove peripheral proteins from membranes.[77] The distribution of myristoylated proteins between different cellular membranes also is less restricted than for palmitoylated proteins, which associate preferentially with the plasma membrane. Analysis of the subcellular distribution of myristoylated proteins has revealed their presence in many intracellular membrane systems.[134]

The apparent absence of myristate on transmembrane glycoproteins and its presence on cytosolic proteins as well as membrane proteins that face the cytoplasm supports the notion that NMT does not participate in modification of proteins transported from the rough endoplasmic reticulum through the Golgi apparatus. The cotranslational nature of this modification also suggests that this enzyme may be associated closely with ribosomes actively involved in polypeptide elongation. In this respect, NMT may exhibit similarities with the *N*-acetyltransferases that transfer acetate to newly synthesized polypeptides.

The identification of myristoylation as a cotranslational modification allows several predictions to be made concerning the molecular events involved in this modification. The predicted sequence of events involved in myristoylation is summarized in Fig. 2. Cotranslational myristoylation of amino terminal glycine residues requires removal of the initiator methionine, and possibly other amino terminal residues. For those myristoylated proteins in which complete amino acid sequence information is available from corresponding cDNAs, the glycine residue that serves as the acceptor is penultimate to the *N*-terminal methionine.[3,22,43,82,104] Cleavage of the initiator methionine is a common modification catalyzed by an aminopeptidase after elongation of the first 30–40 amino-terminal amino acids, while the nascent polypeptide is attached to the ribosome.[83,84] In many proteins, the new amino terminus is rapidly acetylated by a ribosome associated *N*-

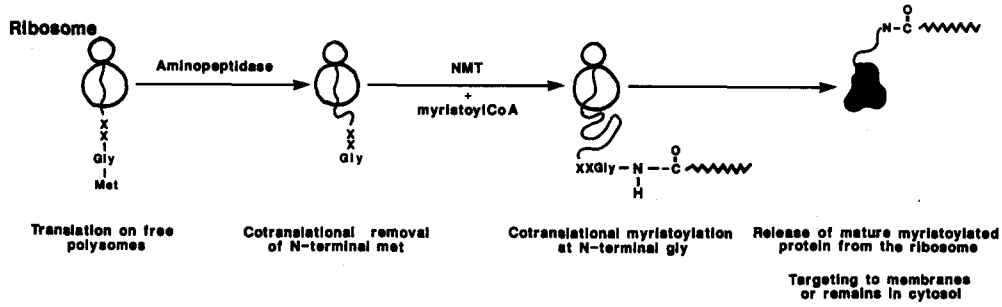


FIG. 2. Biogenesis of myristoylated proteins. Myristoylated proteins are translated on free polysomes in the cytosol where they are acylated cotranslationally using myristoyl-CoA as fatty acyl donor. Acylation is preceded by removal in the *N*-terminal methionine, a reaction catalyzed by aminopeptidase(s). After completion of translation, myristoylated proteins are transported to specific cell membranes or may remain in the cytosol.

acetyltransferase.[12,128] Association of NMT with ribosomes would allow immediate access to newly synthesized polypeptide chains.

It should be emphasized that despite the demonstration that myristoylation can occur cotranslationally, the possibility remains that some myristate-containing proteins also may be acylated very soon after completion of polypeptide chain biosynthesis. That myristoylation may occur with subtly different kinetics is suggested by the observation that labeling of $BC_3H1$ cells with $^3$H-myristate for short periods results in visualization of three major myristoylated proteins, whereas a labeling period of 4 hr reveals 20 or more myristoylated proteins.[77]

Because myristoylation can be reconstituted *in vitro* with artificial acceptor peptides,[125–127] this modification is not coupled obligatorily to polypeptide elongation. The lack of dependence of myristoylation on polypeptide elongation *in vitro* resembles *N*-linked glycosylation and acetylation, both of which occur cotranslationally and have been reconstituted *in vitro* with artificial acceptor peptides containing the appropriate enzyme recognition sequences.[37,132]

Although glycine at position 2 serves as the site for myristoylation in all proteins examined to date, the possibility remains that some proteins may be myristolyated at an internal glycine residue after proteolytic cleavage of a newly synthesized polypeptide. The possibility that myristate may be linked through an amide bond to amino acids other than glycine also has not been formally ruled out.

### 3. *Functions for Myristoylation*

Studies of $p60^{src}$ have provided strong evidence for a role of myristoylation in targeting otherwise soluble proteins to the plasma membrane. $p60^{src}$ is synthesized in the cytosol, myristoylated at its amino-terminus, and transported to the plasma membrane 5 to 15 min after synthesis.[58] Point mutations at the amino terminus of *src* abolish myristoylation and prevent membrane binding.[25,50] Because myristoylation precedes association of the wild type protein with the plasma membrane, it can be concluded that this modification is indeed required for targeting to the membrane. It is interesting to note that mutations that abolish myristoylation of $p60^{src}$ do not interfere with intrinsic tyrosine kinase activity of the protein, but inactivate its transforming activity.[18,25,50] The latter observation suggests that transformation by $p60^{src}$ may require phosphorylation of critical substrates within the plasma membrane that are inaccessible to the enzyme in the cytoplasm. Attempts to identify such substrates, however, have thus far been unsuccessful. Kamps *et al.* reported that nonacylated mutants of $p60^{v\text{-}src}$ do not serve as substrates *in vivo* for phosphorylation by protein kinase C,[17] in contrast to their myristoylated counterparts.[51] These results provide a clear example of a defined protein–protein interaction dependent upon myristoylation.

Studies of viral gag proteins also support the notion that myristoylation influences the interactions of proteins with membranes. Deletion or mutation of the *N*-terminal glycine residue of the Maloney murine leukemia virus $Pr65^{gag}$ prevents myristoylation of the polyprotein precursor.[90] The nonmyristoylated *gag* protein fails to associate with membranes and does not assemble into virions. Inhibition of myristoylation of the Mason-Pfizer monkey virus *gag* polyprotein precursor, due to mutation of the *N*-terminus, also prevents its proteolytic processing.[93] Cells infected with these mutant viruses accumulate A-type virus particles within the cytoplasm, but they do not associate with the plasma membrane or bud.

Using mutants of $p21^{ras}$, Aaronson and coworkers investigated whether the type of fatty acylation influenced the function of the protein within the plasma membrane. Fusion of the coding sequences for the 15 terminal amino acids of $p60^{src}$ in frame to the initiation codon of a $p21^{ras}$ cDNA, that contained a deletion of the carboxy-terminal site for palmitoylation resulted in the synthesis of a p21 polypeptide in transfected cells that underwent myristoylation and targeting to the plasma membrane.[57] Whereas the nonacylated C-terminal deletion mutant failed to induce the transformed phenotype, the

myristoylated chimeric protein induced a phenotype indistinguishable from that of wild type *ras*. Moreover, the wild type and chimeric *ras* proteins both led to increased production of diacylglycerol indicating that the different mechanisms for targeting to the plasma membrane did not influence the function of the protein.

The ability of myristate to direct soluble proteins to membranes was elegantly demonstrated by Hanafusa and coworkers who constructed chimeric proteins by fusing a chimpanzee α-globin cDNA to a sequence encoding the amino terminal 14 residues of $p60^{src}$.[86] Expression of these fusion proteins in fibroblasts resulted in myristoylation and membrane association of globin. While it appears safe to conclude that myristoylation can target an otherwise soluble protein to membranes, the mechanism for selective targeting of these proteins to a specific membrane system is less clear. The myristate moiety must function in a more specific manner than simply serving as a hydrophobic anchor that interacts with the nearest membrane bilayer. If this were the case, one would expect to observe a more homogeneous distribution of acylproteins between cellular membranes, rather than their localization to specific membrane systems such as the plasma membrane. One potential mechanism to explain the specificity of targeting of myristoylated proteins to specific membrane systems might involve myristoyl-protein receptors on membranes that recognize and bind appropriate myristoylated proteins from the cytosol. This type of mechanism might require myristate specifically for recognition by its appropriate receptor. In this regard, it has not yet been determined whether other fatty acids at the *N*-terminus can substitute for myristate. Myristoylated proteins also might associate with carrier proteins in the cytosol that maintain their solubility and direct the proteins to a specific membrane. $p60^{src}$, for example, is transported to the plasma membrane within minutes following its release from the ribosome. During its brief transit through the cytosol, $p60^{src}$ is associated with two cellular proteins: p89 and p50.[15,24] Whether these proteins play some role in targeting to the plasma membrane is unclear.

It remains to be determined whether a myristate moiety is sufficient to anchor an otherwise soluble protein to a membrane in the absence of other membrane proteins to stabilize the interaction or whether the lipid group is simply necessary, but not by itself sufficient, for membrane binding. Studies using artificial membrane vesicles could be employed to address this question.

The functions, if any, for myristoylation of soluble proteins remain enigmatic. At present, the identities of the major cytosolic myristoylated proteins are unknown, which makes the determination of the function of their covalent lipid groups difficult. It is tempting to speculate that these proteins may represent enzymes involved in lipid metabolism or carrier proteins that interact with lipids. Perhaps the NMT or other protein acyltransferases may themselves be myristoylated.

## III. PROTEINS MODIFIED BY GLYCOPHOSPHOLIPIDS

### A. *Characteristics of Proteins Containing Glycophospholipids*

Another class of lipid-containing proteins that is receiving increasing attention is the glycosyl-phosphatidylinositol (PI)-linked glycoproteins. Proteins of this type were identified, initially, following the observation that a PI-specific phospholipase C (PIPLC), caused release of several cell surface proteins from intact cells.[47,60,111] While these results suggested the possibility that such proteins might be anchored to membranes via a phospholipase C-sensitive lipid, there was no evidence at that time to suggest that such a mechanism existed for attachment of proteins to the exterior of cells. Subsequent studies by Ferguson and Cross revealed that the variant surface glycoprotein (VSG) from the parasitic protozoan Trypanosoma brucei was attached to the surface of infected cells through a unique membrane anchor, the structure of which was deduced by a series of detailed chemical and enzymatic analyses.[30,33] Since the initial discovery of the glycosyl-PI modification on VSG glycoprotein, a wide range of proteins have been found to be released from membranes by PIPLC, and have, therefore, been speculated to undergo this type of processing.[26,59] Proteins believed to contain covalent glycosyl-PI are shown in Table 3.

TABLE 3. Proteins Modified by Glycosyl Phosphatidylinositol

| Protein | Reference |
|---|---|
| Thy-1 glycoprotein | 59, 62, 129 |
| Variant surface glycoprotein of trypanosomes | 33 |
| Acetylcholine esterase | 35 |
| Alkaline phosphatase | 63 |
| 5′-nucleotidase | 61 |
| Neural cell adhesion molecule-120 | 42 |
| Plasmodium falciparum transferrin receptor | 40 |
| Plasmodium falciparum p195 surface antigen | 39 |
| TAP glycoprotein | 92 |
| Trehalase | 122 |
| Decay accelerating factor | 71 |
| Scrapie ($PrP^{Sc}$) and cellular ($PrP^{c}$) prion | 116 |
| Lymphocyte function-associated antigen 3 | 29 |

## B. *Structures of Glycophospholipid Anchors*

The complete structures of the membrane anchoring domains of the majority of the proteins in Table 3 have not yet been determined; however, metabolic labeling studies and PIPLC sensitivity of these proteins support the conclusion that they are linked to membranes via a glycosyl-PI anchor. Detailed structural information has been obtained for the membrane anchor of VSG.[30,32,33,46] The structure of the glycosylated-PI modifying group that anchors VSG to membranes is shown in Fig. 3. Within this complex molecule are several important structural constituents. (1) The glycolipid is linked to the polypeptide

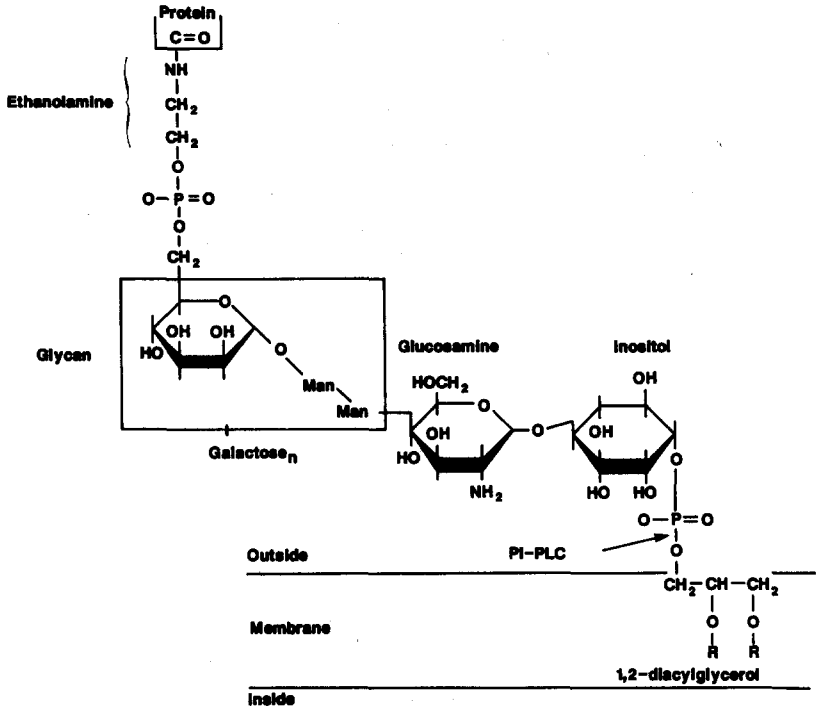


FIG. 3. Structure of the glycosyl-PI anchor.[26] This structure was determined by detailed chemical analysis of the membrane anchor of VSG. Indirect evidence suggests that other PI-linked glycoproteins contain similar structural components, but may exhibit subtle variations. The anchor contains 3 regions: (1) a phosphatidylinositol moiety is embedded in the bilayer and provides the linkage to the membrane, (2) a glycan that can exhibit heterogeneity among different proteins is linked to phosphatidylinositol through a glycosidic linkage with a glucosamine, (3) an ethanolamine is linked through its amino group to the α-carboxyl of the C-terminal amino acid, which in the case of VSG is aspartate. PI-PLC removes the diacylglycerol moiety from the PI anchor releasing the polypeptide and its associated glycosyl-inositol phosphate into the extracellular milieu.

through an amide bond formed between the carboxy-terminus and the ethanolamine group. (2) The ethanolamine appears to be linked by a phosphodiester bond to an oligosaccharide. The structure of the carbohydrate is the most poorly characterized aspect of the anchor and varies in the degree of galactosylation in different proteins. (3) The glucosamine group is not substituted, which is an unusual situation among eucaryotic protein-linked glycans. (4) The glucosamine is glycosidically linked to the myo-inositol portion of PI that serves as the site for membrane attachment. The fatty acids contained within the diacylglycerol moiety appear to vary between anchors on different proteins: VSG contains exclusively myristate,[30,32] Thy-1 has primarily stearate,[129] and human erythrocyte acetylcholine esterase has mainly palmitate.[96]

The membrane anchoring structure of Thy-1 also has been determined in considerable detail. Trypsin digestion releases the carboxy terminal cysteine of Thy-1 in covalent association with ethanolamine, phosphate, mannose, glucosamine, galactosamine, inositol and stearic acid in molar ratio of 2:2:1:1:1:1:1, respectively, in addition to smaller amounts of a variety of other fatty acids.[129] The globular form of acetylcholine esterase (AChE), which associates with membranes through hydrophobic interactions, has been isolated from the electric organ of *Torpedo californica* and from human erythrocytes.[35,38] The globular AChE from electric organ contains stoichiometric amounts of myoinositol and can be recovered completely from membranes by PIPLC digestion.[35] Erythrocyte AChE contains ca. 2 moles of fatty acid per mole of enzyme, in addition to an ethanolamine residue linked through an amide bond to the carboxy-terminus.[38] In the case of this protein, however, only about 10% is susceptible to release by PIPLC digestion. The basis for this difference in behavior remains to be determined. Decay accelerating factor (DAF) also has been demonstrated through detailed chemical analysis to contain ethanolamine and glucosamine and to be released from membranes by PIPLC.[27] The susceptibility of this protein to PIPLC also varies between cells, with 80%, 53%, and 10% being released from peripheral lymphocytes, neutrophils and erythrocytes, respectively.[27]

### C. *Biogenesis of Proteins Containing Glycophospholipid Anchors*

The timing and subcellular sites of addition of glycosyl-PI to proteins have been examined in detail within VSG infected cells. The steps involved in this process are shown in Fig. 4. Polypeptides destined to acquire PI anchors are translated on membrane-bound polysomes in the rough endoplasmic reticulum.[47,60,111] Pulse-chase analysis indicates that the membrane anchor is attached within 60 s following completion of translation of the polypeptide chain.[5,31] Analysis of full length VSG cDNAs has shown that these proteins are synthesized initially as precursor proteins, which contain a hydrophobic carboxy terminal domain that is absent from the mature protein.[13] Similarly, DNA sequence analysis of Thy-1 predicts a 31 amino acid hydrophobic tail that is removed during maturation of the polypeptide.[108] The length of the carboxy-terminal peptide that is removed from each of these proteins is not of sufficient length to span the bilayer and the ribosomal subunits, indicating that addition of the membrane anchor must occur after completion of translation. The rapid attachment of glycosyl-PI to the carboxy terminus is preceded by proteolytic cleavage of the hydrophobic domain. Amino acid sequence analysis of proteins that acquire membrane anchors has failed to reveal obvious concensus sequences either for proteolytic processing or for attachment of glycosyl-PI. The actual carboxy-terminal residue to which the glycolipid anchor it attached also varies between proteins,[59] in contrast to the amino acid specificity observed for attachment of palmitate or myristate to proteins. The attachment of this complex structure to the carboxy-termini of this diverse group of proteins, however, strongly suggests that some recognition sequence must be present within these polypeptides to direct their appropriate processing. Rather than a specific sequence, the actual signal for processing may be conformational, as in the case of the signals for myristoylation and for *N*-terminal cotranslational insertion of nascent polypeptide chains through membranes. The multistep nature of this form of processing also suggests the involvement of at least two enzymes, a carboxy-terminal

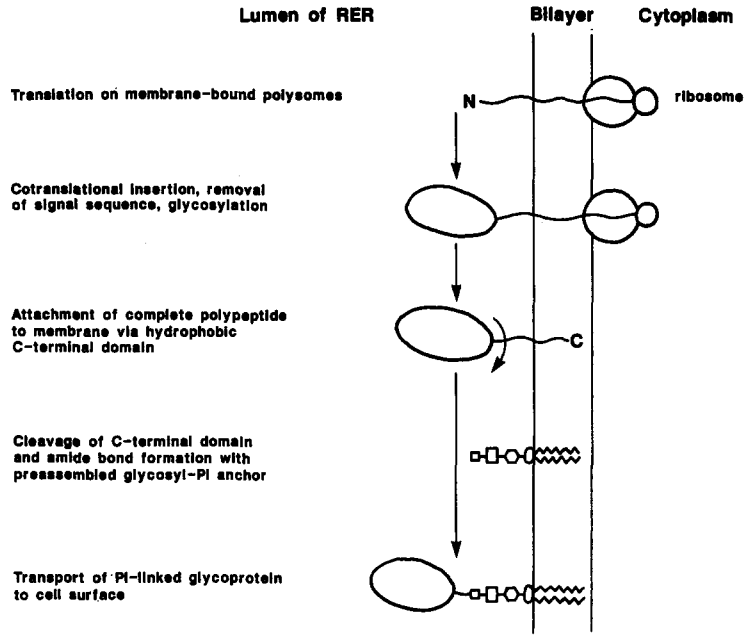


FIG. 4. Biogenesis of proteins containing a glycosyl-PI anchor. The scheme depicted above is based on studies of VSG; however, a similar sequence of events appears likely for other PI-linked glycoproteins. The nascent polypeptide chain is translated on membrane bound ribosomes on the rough endoplasmic reticulum (RER). Translational insertion through the bilayer, removal of the *N*-terminal signal sequence, and *N*-linked glycosylation occurs in the same manner as for other transmembrane glycoproteins. Following termination of translation, a carboxy-terminal hydrophobic domain of ca. 20 residues anchors the newly synthesized polypeptide to the membrane and prevents its release into the lumen of the RER. The hydrophobic C-terminal domain is then removed and the newly exposed C-terminus is linked through an amide bond to a preassembled glycosyl-phospholipid precursor. This anchor tethers the otherwise soluble protein to the membrane and carries it via the secretory pathway through the Golgi apparatus to the cell surface.

peptidase and the glycosyl-PI transferase. Little is known as yet, however, of these enzymes.

Attachment of the glycolipid moiety to newly synthesized proteins is unaffected by inhibition of *N*-linked glycosylation with tunicamycin,[4] but is arrested almost immediately following inhibition of protein synthesis with cycloheximide.[5,31] This extremely rapid inhibition after blocking protein synthesis suggests that the enzymes involved in this modification are restricted to the endoplasmic reticulum. These results also suggest that cells do not contain a pool of proteins that can be modified by glycolipids at different times following synthesis. Monensin, an inhibitor of transport through the Golgi apparatus, also does not affect glycolipid attachment, but may prevent carbohydrate modification of the lipid backbone.[4]

### D. *Functions for Glycophospholipid Anchors*

In contrast to the other forms of protein lipidation, in which the functions for the modification are often vague, the functions of glycosyl-PI are relatively clear. Proteins modified by this mechanism are attached to membranes solely via the PI moiety. As discussed above, removal of the covalent membrane anchor by PIPLC results in release of these proteins into the extracellular milieu. In addition to its obvious role in binding otherwise soluble proteins to the membrane, PI anchors may serve more subtle functions. Because proteins linked to the bilayer do not contain transmembrane domains that interact with other membrane proteins or with the cytoskeleton, PI-containing proteins are relatively unrestricted in their mobility and would be expected to migrate rapidly within the bilayer. Indeed, fluorescence photobleaching studies indicate that the mobile fraction

of Thy-1 exhibits a relatively high diffusion coefficient,[48] although it has not yet been determined whether there is any relationship between the nonmobile fraction and the PIPLC-resistant forms of Thy-1. Alkaline phosphatase (AP) also exhibits high lateral mobility in normal cells and in cells transfected with the AP cDNA.[73] The maximum rate of diffusion of AP in the membrane has been reported to be closer to the diffusion rate of membrane lipids than of most integral membrane proteins. A PI anchor may, thus, provide a mechanism for rapid mobility of specific proteins within membranes.

An additional function for a PI anchor might be to provide the cell with a mechanism for rapid release of proteins from the cell surface in response to external stimuli. In light of the complexity of the membrane anchor, it is tempting to speculate that this structure also might serve roles in addition to providing a hydrophobic tail. Treatment of membranes with PIPLC, for example, releases PI-linked proteins with a tail containing an oligosaccharide, ethanolamine and inositol phosphate and leaves behind diacylglycerol within the membrane. The portion of the PI anchor retained by the released protein might participate in recognition or adhesion events at the cell surface, whereas diacylglycerol, a natural activator of protein kinase C, might transduce signals across the plasma membrane. The potential involvement of glycosyl-PI anchors in these types of cellular processes will provide an interesting avenue for future research.

## IV. PROSPECTS

Protein lipidation clearly represents a widespread type of covalent modification that plays an important role in directing specific proteins to membranes. Based on metabolic labeling studies, it appears that only a minor fraction of the proteins that contain lipid have been identified. Considering the large number of regulatory proteins that already have been shown to require covalent lipid to exert their appropriate functions, it will be particularly interesting to establish the identities of additional lipid-containing proteins. In the case of proteins modified with covalent fatty acids, it will also be important to define the mechanism(s) involved in lipid-mediated targeting to specific membranes and to determine whether specific fatty acids confer specialized properties on the proteins to which they are attached. Similarly, for those proteins that contain glycosyl-PI anchors, it will be interesting to investigate whether the subtle structural differences in the anchors on different polypeptides reflect specialized roles for these modifying groups. Studies of the role of PI-PLC and the products of hydrolysis of PI-containing glycoproteins in inter- and intracellular signalling also may yield additional insights into the functions for protein lipidation. Finally, much remains to be learned about the enzymology of protein lipidation.

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